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(57) Abstract

The invention provides a recombinant nucleic acid construct comprising: (i) a mammalian myosin heavy chain promoter; (ii) nucleic acid encoding a signal sequence; and (iii) nucleic acid encoding a functional blood clotting protein; the elements (i)-(iii) being operably linked to provide for expression of the functional protein. The construct is useful for the treatment of diseases such as haemophilia by introducing it into muscle cells. The activity of the promoter can be enhanced by exercise or stimulation of the muscle cells.

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GENE THERAPY FOR HAEMOPHILIA

The present invention relates to gene therapy, and to DNA vectors for the use in such treatment.

Methods of treating genetic diseases by gene therapy have been proposed. Genetic diseases which have been the subject of preliminary clinical trials include cystic fibrosis (CF) and adenosine deaminase (ADA) deficiency.

Haemophilia A is an X-linked genetic disease caused by a defect in the gene coding for the blood clotting protein, Factor VIII. The incidence of haemophilia is about 1 in 5,000 of the male births. Sufferers from haemophilia are unable to clot blood properly at the site of wounds. In addition to the dangers this poses for the treatment of open cuts, the inability to clot blood properly causes damage to joints and to internal tissues, eg muscles.

Treatment of haemophilia A is possible by the administration of Factor VIII. Until recently, Factor VIII preparations had to be prepared by concentration of blood donations which was problematic in that the preparations could be contaminated with infectious agents such as Hepatitis B virus, Hepatitis C virus or HIV. The gene for Factor VIII has been cloned (see for example Vehar et al, Nature, 1984, 312;337) and this has allowed the production of recombinant Factor VIII. Although this allows supplies of Factor VIII protein which are of higher purity than blood concentrates, the exogenous supply of Factor VIII to a patient still means that repeated doses are required throughout the lifetime of a patient, which is inconvenient and expensive.

Other forms of haemophilia include haemophilia B, caused by a defect in the gene coding for factor IX.

A number of serious ethical and medical problems arise when considering the treatment of diseases by gene therapy. The introduction of exogenous DNA into the cells of the patient may have long term effects which cannot be foreseen at present. For example,

many cancers are known to be caused by rearrangement of genomic DNA, and thus any introduction of DNA into a patient which caused rearrangements or disruption of the genome could be deleterious to the patient. A further problem is identifying suitable tissues for the introduction of exogenous genes and in controlling the expression of such genes.

It has been reported that plasmid DNA injected into the muscle of rodents and other mammals is taken up by the cells of such animals but the injected DNA does not integrate into the cells. The direct transfer of DNA into muscle cells in this manner has been proposed as a means of effecting somatic gene therapy (Wells and Goldspink, FEBS Letters 1992, 306;203-205) although in order to develop this proposal a number of practical difficulties still remain. For example, although muscle cells can take up DNA, muscle cells are not known to be natural exporters of proteins. Furthermore, levels of expression of reporter genes introduced into primate muscle cells have been low and such levels have been considered unpromising for the development of gene therapy (Jiao et al, Hum. Gene Therapy, 1992, 3;21-33). International patent application WO93/09236 describes myogenic vector systems for the expression of a nucleic acid sequence in myogenic tissue.

The present invention seeks to address the above mentioned problems by providing a DNA construct for gene therapy of haemophilia and other blood clotting disorders. We have identified a class of muscle specific promoter sequences which can be linked to a gene encoding a functional human blood clotting protein carrying a signal sequence. We have surprisingly found that this enables the functional protein to be exported from the muscle cell, thus permitting delivery of the protein, via the bloodstream, to a desired site of action. Furthermore, we have found that the use of a muscle specific promoter provides a steady constitutive level of expression which allows an effective amount of protein to be produced. The level of expression can be enhanced by stimulation of the muscle cells. This enables the output of the engineered gene product to be increased mimicking the natural situation with regard to clotting factor production (flight and fright response).

Thus, in one aspect, the present invention provides a recombinant nucleic acid construct comprising:

(i) a mammalian myosin heavy chain promoter;

(ii) nucleic acid encoding a signal sequence; and

(iii) nucleic acid encoding a functional blood clotting protein;

the elements (i)-(iii) being operably linked to provide for expression of the functional protein.

The myosin heavy chain promoter is one which will enable selective expression of the construct in muscle cells. This means that the construct will not function to any significant extent, if at all, in other cells types, eg liver or epithelial cells, in comparison to the levels of expression in muscle cells, especially skeletal muscle cells. Suitable promoters include the α-myosin and β-myosin heavy chain promoters.

A preferred mammalian myosin heavy chain promoter is the β-myosin heavy chain promoter. The human, pig and rabbit forms of this promoter have been obtained and these are set out below. The promoter region of the human and porcine β-myosin heavy chains are shown numbered upstream from the presumed start of transcription. The rabbit sequence can be obtained as described in Cribbs *et al*, J. Biol. Chem. 264; 1989, 10,672-10,678. Alternatively, it may be made synthetically based upon the sequence shown. The other mammalian forms of these promoters can be obtained in an analogous manner. For example, fragments of the human promoter sequence given below can be synthesised and used as probes to probe a genomic DNA library made from humans or another species of mammal. The probe will be used under conditions which will enable homologous sequences to hybridize. Suitable hybridization conditions can be determined by reference to standard manuals, eg. Sambrook *et al*, Molecular Cloning (1989, Cold Spring Harbor, N.Y).

The human β-myosin heavy chain promoter is set out in Seq. ID No.3. There is a "TATA" box at nucleotides 871-876 of the sequence and the start of transcription has been determined to be at the G nucleotide at position 906. The porcine (pig) β-myosin heavy chain promoter is set out in Seq. ID No.4. The "TATA" box region is highly

homologous to the human sequence of Seq. ID No.3, and is at positions 822-827 of Seq. ID No.4. The start of transcription has been determined to be at the A nucleotide at position 845. The rabbit B-myosin heavy chain promoter is set out in Seq. ID No.5. A "TATA" box is found at positions 653-658 and transcription starts a short distance downstream of this. The start of transcription from all the above promoters may be confirmed or determined by standard techniques such as S1 mapping or primer extension.

In order to achieve selective expression of a construct according to the invention, it is desirable to use a promoter region from the "TATA box" (usually found about 30 nucleotides from start of transcription) upstream to include sequence elements responsible for specificity of expression in muscle cells. The myosin heavy chain promoter will desirably include at least all the nucleotides upstream (5') of the TATA box to about the 400th, eg 500th, 600th, 700th, 800th, 900th or further nucleotide 5' to the start of transcription. It is also preferred to include the TATA box together with the native sequence downstream of the TATA box to at least the start of transcription.

Desirably, the promoter is modified to remove regions which control specificity of expression in different types of muscle cells. Desirably, this is achieved by truncating the promoter at about between 500 to 1000, preferably about 700 to 900, for example about 750 to 850 nucleotides upstream from the start of transcription of the myosin gene from which the promoter is derived. We have found that β-myosin heavy chain promoters truncated in this way have the ability to be expressed in a variety of muscle types (eg. skeletal and cardiac). Alternatively the promoter may be modified to delete or alter specific regions responsible for this level of tissue specificity. For example, site-directed mutagenesis could be used to modify the sequence of regions of the promoter, and such promoters tested using constructs of the invention for their ability to express genes in both skeletal and cardiac muscle cells.

Although β-myosin heavy chain promoters corresponding to naturally occurring muscle specific promoter DNA are preferred, the invention also includes modified myosin heavy chain promoter sequences which are capable of selectively hybridizing to the

naturally-occurring sequences. A promoter sequence capable of selectively hybridizing to a naturally-occurring myosin heavy chain promoter sequence will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the promoter region or fragment thereof over a region of at least 20, preferably at least 30, for instance 40, 60, 100 or 500 or more contiguous nucleotides. Such promoter regions are included within the scope of the invention and are regarded as mammalian myosin heavy chain promoters. Desirably, these modified sequences still retain the tissue specificity of their natural counterparts.

Although it is preferred that a construct according to the invention is DNA, it may also be RNA or modified nucleic acid. The nucleic acid may contain modifications in its backbone and possibly additions at either the 5' or 3', or both, ends of the molecule (in the case of linear, as opposed to circular, constructs). This may assist in prolonging the life of the DNA when taken up by muscle cells which may enhance the potency of the construct. Known modification to DNA molecules include the provision of methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule.

The signal sequence may be any mammalian signal sequence which can be made to export protein from muscle cells. We have found that the 19 amino acid factor VIII signal sequence set out in Seq. ID. No.1 will enable expressed proteins to be exported from muscle cells. DNA encoding this sequence, for example the DNA sequence of Seq. ID. No.1 may be used in the construct of the invention. Other examples of signal sequences which may be used include the signal sequences of insulin-like growth factors I (Jansen et al, Nature, 1984, 306;609-611) or II (Bell et al, Nature, 1984, 310;775-777). Other signal sequences may be obtained and tested for their ability to drive the export of the functional protein from muscle cells. It is also preferred that the signal sequence is capable of being cleaved from the functional protein during or following the export process so that the functional protein will appear in its native form within the vascular compartment.

Blood clotting proteins include coagulant and anti-coagulant proteins involved in physiological haemostatic mechanisms.

One preferred blood clotting protein is factor VIII. The nucleic acid encoding Factor VIII protein is preferably the human Factor VIII cDNA or an active fragment thereof. The amino acid and DNA sequence may be obtained by reference to Wood et al (Nature, 1984, 312;330-337.) The entire coding region of the cDNA from nucleotide 1 to nucleotide 7040 (as specified by Wood et al, ibid) may be used. Alternatively, a modified cDNA lacking the B domain (amino acids 712-1648 of Wood et al, ibid) may be used.

The factor VIII gene contains a signal sequence to direct export of the factor VIII protein from a cell, and it is preferred that the signal sequence of the construct is the native factor VIII signal sequence.

Other blood clotting proteins such as factor IX (the deficiency of which causes haemophilia B) may also be encoded by constructs according to the invention. The coding sequence for factor IX may be obtained by reference to Anson et al, Nature, 1985, 315;683-685.

Factor VII may also be expressed in a construct of the invention. The factor VII sequence may be obtained by reference to Hagen *et al*, Proc. Natl. Acad. Sci., 1986, 83; 2412-2416.

Further blood clotting proteins which are preferred include Von Willebrand factor (the gene for which may be obtained by reference to Mancuso et al, J. Biol. Chem, 1989, 264; 19514-27), Factor X (Leytus et al, Biochemistry, 1986, 25; 5098-5102), Factor XI (Asakai et al, Biochemistry, 1987, 26; 7221-28), Factor V (Jenny et al, Proc. Natl. Acad. Sci. USA, 1987, 84; 4846-4850), Protein C (Proc. Natl. Acad. Sci. USA, 1985, 82; 4673-77), Protein S (van Amstel et al, Biochem., 1990, 29; 7853-61) and anti-thrombin III (Chandra et al, Proc. Natl. Acad. Sci. USA., 1983, 80; 1845-48). Genes, in the form of genomic DNA, cDNA or engineered mini genes, encoding these and

other blood clotting proteins may be used to produce constructs according to the present invention. Engineered mini genes include cDNA sequences intb which one or more introns have been introduced or genomic DNA sequences which have been modified to remove one or more introns.

"Operably linked" refers to a juxtaposition wherein the muscle specific regulatory element and nucleic acid encoding the signal sequence and the functional protein are in a relationship permitting them to function in their intended manner. A promoter sequence according to the invention "operably linked" to the coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the promoter and any other control sequences.

The construct may also contain a poly-adenylation signal operably linked 3' to the nucleic acid encoding the functional protein. Desirably there will be a region of 3' untranslated (3'-UT) sequence between the coding sequence and the poly-adenylation signal. Typically, the 3'-UT region will be about from 50 to 1000 base pairs. Such a sequence may be, for example, the 3'-UT sequence of the gene which is being expressed. Alternatively it may be a 3'-UT sequence from a muscle specific gene, including the β-myosin heavy chain gene itself. It will also be possible to use other mammalian or viral 3'-UT sequences.

Optionally, the constructs of the invention may also contain an enhancer for the promoter. Suitable enhancer elements include a myosin light chain enhancer sequence. One such sequence is the 900 base pair myosin light chain enhancer element as described by Donoghue *et al*, Genes and Development, 1988: 2; 1779-1790. The enhancer element may be inserted into a construct of the invention either 3' or 5' of the promoter and gene which are to be expressed. It is preferred that the enhancer is 3' to the gene and poly-adenylation signal.

The constructs according to the invention may be incorporated into vectors, for example, plasmid, virus or phage vectors provided with an origin of replication. The vector may contain one or more selectable marker genes, for example an ampicillin

resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a vector for mammalian cells.

It is preferred that the construct is incorporated into a plasmid vector, since it has been found that covalent closed circle (CCC) plasmid DNA can be taken up directly by muscle cells but that the DNA does not integrate into the genomic DNA of the cells.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the replication and expression of vectors according to the invention. The cells will be chosen to be compatible with the vector and may for example be bacterial, yeast, insect or mammalian.

While it is possible for the nucleic acid constructs of the invention to be administered alone it is preferable to present them as pharmaceutical formulations. The formulations of the present invention comprise a nucleic acid construct according to the invention, together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier or diluent will preferably be such that the composition is suitable for injection into skeletal muscle of a patient, for uptake of the construct or vector by the skeletal muscle cells. The carrier or carriers must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients thereof, for example, liposomes. Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoylphosphatidylethanolamine (DOPE), and those comprising 3β[N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood

components or one or more organs. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water, for injections, immediately prior to use. Injection solutions and suspensions may be prepared extemporaneously from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question. Of the possible formulations, sterile pyrogen-free aqueous and non-aqueous solutions are preferred.

The invention also provides constructs, vectors and compositions according to the invention for use in a method of treatment of a mammal, including man.

The invention also provides a method of treatment of a human or animal subject, which subject is suffering from a deficiency in a functional protein, which comprises administering to the skeletal muscle of said subject an effective amount of a construct or vector according to the invention encoding said functional protein.

Although the effective amount of a construct or vector will ultimately be at the dose of the physician, taking into account the nature of the form of haemophilia being treated and the condition of the patient, suitable dose are in the range of from about 10 μg to about 10 mg of DNA per kg of muscle tissue, eg. from about 100 μg to about 5 mg, eg. from about 1 mg to about 2.5 mg per kg of muscle tissue.

The DNA may be administered in a single dose or in divided multiple doses. If the DNA is administered in divided doses, the doses may be administered to different muscle tissues in the body.

The doses may be administered sequentially, eg. at daily, weekly or monthly intervals, or in response to a specific need of the patient, eg. following injury or other physical

trauma.

Preferred routes of administration are oral delivery and injection, typically intramuscular injection. Injection of the vaccine composition into the skeletal muscle of the human or animal subject is particularly preferred. Another mode of delivery of a vaccine composition according to the invention is by a biolistic or "particle gun" method.

The following examples illustrate the invention.

Example 1.

Production of a Factor VIII construct

A rabbit β-Myosin heavy chain promoter of 781 bp (Fig.1 and Cribbs *et al.*, J Biol Chem, 1989, 264:10672-10678) was cloned into the Hind III site of the plasmid vector pUC 19, with the 5' end of the promoter facing away from the poly cloning site of the vector, leading to the clone pPR. Two complementary overlapping 45mers (oligonucleotides) were designed to fulfil the requirements for a functional polyA tail signal (McLauchlan *et al.*, Nucleic Acids Research, 1985, 13:1347-1368) with homologies to the human α-globin polyA signal sequence (Liebhaber *et al.*, Proc Natl Acad Sci USA, 1980, 77: 7054-7058). When annealed the 45mer, designed to have two sticky ends, could be subcloned into pPR cut with Sal I and Bam HI, thus leading to clone pPRPAS. Clone pPRPAS-E9 was obtained by inserting a mouse myosin light chain enhancer element of 900 bp (Donoghue *et al*, Genes & Development, 1988, 2:1779-1790) into the Bam HI site of pPRPAS. A cDNA sequence from blood clotting factor VIII spanning nucleotides 1-7040 (Wood *et al.*, Nature, 1984, 312:330-337; cDNA clone obtained from this group) was finally inserted into the Sal I site, thus leading to pPRF8PAS-E9. The construct spans approximately 11,500 bp. Large scale preparations were produced by transfection into competent *E Coli* (SURE Cells, Stratagene) and standard purification methods.

Digests with specific restriction enzymes give rise to the following DNA fragment sizes [kb]:

Eco RI:	0.35,	1.1,	1.7,	3.7,	4.68	(5 fragments)
Bam HI:	0.9,	2.3,	2.9,	5.5		(4 fragments)
HindIII:	0.4,	0.65	0.8,	1.25	8.4	(5 fragments)

Example 2

From a library of 5' untranslated regions from α and β myosin heavy chain gene constructs containing the different myosin heavy chain promoters from rabbit have been cloned, as described by Cribbs *et al*, J. Biol. Chem, 1989: 264: 10672-10678. In a truncated form, the slow type 1 (β cardiac) promoter has a general specificity for skeletal muscle yet can still be induced by increased activity.

The β-cardiac promoter was tested in bandshift assay (gel retardation assays) with nuclear protein extracts from various skeletal muscle tissues from rabbit. The binding pattern of nuclear proteins to specific promoter regions could not detect a difference between soleus and tibialis anterior, making both muscles a candidate for gene expression driven by the cardiac promoter.

To establish the onset of β-cardiac promoter activity as well as its endurance, transfection experiments were carried out *in vitro*. Mouse myoblasts from the cell line C2 were grown to 80% confluence (approx 1.5×10^6 cells) and transfected with 20 μg DNA of a chimeric plasmid, containing the CAT reporter gene drive by the β-cardiac promoter. The same myoblasts were cotransfected with 10 μg pCH101, a β-gal reporter construct driven by a viral promoter. 24 hours after transfection differentiation was imitated by reducing the serum in the culture media from 10% fetal calf serum to 4% horse serum. Cells were harvested 1, 3, 5, 7 and 9 days after differentiation onset. Protein extracts were standardized with a chromographic β-gal assay and tested for

CAT activity. All extracts showed the same amount of CAT activity, thus suggesting the promoter to be activated from the early stages of differentiation through to fully differentiated muscle tubules.

Example 3

Similarly a myosin light chain enhancer sequence has been identified. The 900 base pair myosin light chain enhancer element described by Donoghue *et al*, Genes and Development, 1988: 2: 1779-1790, was tested in an expression vector containing, 5' to 3', the rat MLCI promoter, the CAT reporter gene and the enhancer. Transfections of C2 myoblasts and cotransfections with pCH101 were carried out as described above for the β-cardiac promoter/CAT gene construct. CAT assays revealed high activity of the construct in all differentiation stages. The excision of the enhancer and subsequent transfection with a construct containing only promoter and CAT gene revealed a significantly lower expression compared to the full construct. Thus the enhancer element proved to upregulate expression in the chosen cell system.

Example 4

The construct of Example 1 was transfected into a myoblast cell culture system (C2 cells). 20 μg of plasmid DNA was incubated with myoblast monolayers cultured in 10% foetal bovine serum-DMEM in the presence of 2mM $CaCl_2$ for 16-24 hours. 48 hours after transfection the medium was changed to 4% horse serum-DMEM to induce fusion and differentiation of myoblasts to myotubes. Culture supernatant was harvested and stored for testing. Nine days after transfection all muscle cells were harvested and total RNA isolated by standard methods.

Expression of the factor VIII construct was tested at the level of transcription. Using a reverse transcription polymerase chain reaction, factor VIII mRNA was identified, using primers to exon 14 so demonstrating the presence of factor VIII mRNA in transfected cells. Although a nested PCR was performed for clarity of result, the

expected product was demonstrable after a single round of amplification.

Example 5

A construct analogous to the factor VIII construct of Example 1 was made except that the expression cassette contains 2.46kb factor VII cDNA (sequence as given by Hagen *et al* Proc. Natl. Acad. Sci, 1986: 83: 2412-2416) from nucleotide 1 to nucleotide 2426, cloned into the cassette Sal I site. This construct contains some 5' and 3' untranslated regions of factor VII.

Using this construct and transfecting myoblasts monolayers, as for Example 4, measurable levels of factor VII protein in both cell culture supernatant and cell extract were demonstrated easily. Using a modified VII: Ag ELISA (Diagnositica Stago, Asserachrom VII:Ag) the supernatant showed approximately 20u/dl, and had levels of VII:Ag four to five fold higher than cell extract. These results demonstrate that factor VII:Ag is expressed and secreted by muscle cells into the culture medium. In addition the cell supernatant was tested for biological activity in a one stage prothrombin time based factor VII assay. This assay showed the supernatant to have levels of approximately 40u/dl VII. The apparent discrepancy between the VII:Ag assay and VII clotting assay may be accounted for by the modifications made to VII:Ag assay when being used for analysis of the supernatant. The clotting assay was able to treat the supernatant as any other plasma sample. Control supernatant had a VII activity of 4u/dl.

The demonstration of biological activity of supernatant in a one stage clotting assay demonstrates effective post translational terminal gamma carboxylation of transfected factor VII protein.

50 μg of the factor VII construct of Example 4 in a hypertonic solution (20% sucrose final concentration) was injected directly into quadriceps muscles in two sites. At daily intervals, from day 1 to 7, and at 14 and 21 days, injected mice were sacrificed and

bled by intracardiac puncture. Plasma samples were then assayed for VII:Ag(human VII:Ag is not cross reactive with murine VII:Ag in this assay system). All animal work was performed in duplicate.

One animal (day 5 sacrifice) showed a 4 fold increase in VII:Ag compared to background. This result was confirmed on repeat testing.

Example 7

A B-domainless factor VIII is constructed. The B domain of the factor VIII gene is inhibitory to the expression of factor VIII. Eliminating this domain improves gene expression. The construct is made using analogous methods to those described in Example 1.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Gene Therapy for Haemophilia

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..57

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG CAA ATA GAG CTC TCC ACC TGC TTC TTT CTG TGC CTT TTG CGA TTC      48
Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe
 1               5                  10                  15

TGC TTT AGT                                                      57
Cys Phe Ser
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe
 1               5                  10                  15

Cys Phe Ser
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 935 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTCCTGCTCT CTGCGCATAG GGGGTCCCCA CATCTTTGGA ATCCCAGCCC ACCTTTCCAG 60

GCTACCCTCC ACAGGCCGGG CTCCACTCCC ATCTGCTGAG GTTTCCCACC TTGAATCCTG 120

CTGCCCCGAT TAGCTGTATA ACCTTAAAGA ATGCACTGTC CCTCTCCATT AAATGAAGTG 180

CTTGGATGGA TTGCTAAAGG CCTGTCTGGC TCGGAGGCTT GGTGCCTCAA CACATTGCCT 240

GCTGGTCCAA GGAAATCAGT GCCTGAGCCA GAGTCCCCAT CTCTAAGCTC CATGGTTATT 300

GTTCTTGCCA CCTGGCTAGG AAATGTCCTT CCAGCTGCCC CAGTCTAGCT GCCTCACCCT 360

GGGGCCATGC CCCAACTCTG TCCTACCCTT CTCTGCTGCT GACACTCAGC CCCTTCCCAG 420

CTTCCAGTTG GATACAGGAC CTGGGCCAGG AGAGCAGGGA GGACACTGTG GAAATGCGGC 480

CAGGCCATCA GGGGCCTCGC AGCAGGGGAC TGGAGGGGGA GCAGTGTCCA GGGCCAGAAG 540

TGCCCTGCGG GAGAGCCAGG ACATTGGCTG CCTGTGGTCT TGGTGGTCGT GGTCAGTTCC 600

CTCTCCTGCC AGCTGTGGAA TGTGAGGCCT GGCCTGGGAG ATATTTTTGC TGCACTTTGA 660

GCCACCCCGC CCCCTGGAAC TCAGACCCTG CACAGTCCAT GCCATAACAA TGACGACCAC 720

TTCCAATTGT TTCCTAGCTG GAGAGGCGGG GGAGGGAGCA CTGTTTGGGA AGGGGGGGAG 780

CCTGGGGGAA ATGCTTCTAG TGACAACAGC CCTTTCTAAA TCCGGCTAGG GACTGGGTGC 840

CGTTGGGGGT GGGGGTGCCC TGCTGCCCCA TATATACAGC CCCTGAGACC AGGTCTGGCT 900

CCACAGCTCT GTCCTGCTCT GTGTCTTTCC CTGCT 935

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 886 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATCCTCGCCT CCTTGGACTC CCAGCCCTCC TTCCCAGGCC TCCCTCTTAC AGGCTGGGCT 60

CCATCCCCAT CTGCCGAGCT CTCCTCCACC CCTCCCTTGA ATCTTGCTGC CCTGACCAGC 120

TGCAGGACCT TAAAGAGGGC ATTTCCTCTC TCCGTAAAGT GAGGGACTTG GACCCACTAC 180

TGAGGCCTGT CTGGCTCTGA GGTCCCATGA CCTCAACACA TGGCCAGGCC AGTCCAAGGA 240

AACCAGTGCC CCATCCAGAG TCCCCATTTC TAATCTCCCT AGTCGCTGTT CTTGCCCATC 300

TGGCTGGGAA ATCTTCTTCC AGCCTGCCCC ATGGCCCAAC TCTGCTCTGC TCCTCTGCTG 360

CTGACTCTCA GCCCTTCCCA TCTCACAGTT GGAGGCATGA CCTGGGCCAG GGAGCAGGGA 420

GGACTCTGTG CAAGAGGGCC CAGGCTGGTC CCAGGGGGCA GGGGCCGAGG GCAGCAGTGT 480

CAGGGGCCAG GAGAGCACTG GGGGAAGCCA GGCCCTGCCT GCCCGAGGTC ATGGTGGTCG 540

TGGTCAGTTC CCTCTCCTGC AGCTGTGGAA TGTGAGGCCC GGCCTGGGAG ATATTTTGGC 600

TGTACTTAGA GCCATCCCCG CCCCCTGGAA TCAGACCCTG CTCACTCCAT GCCATAACAA 660

TGACGACCAC TTCCAATTGT TTCCTAGGTA GGAGGGGGTG GGGAGGGGGA GCATGGCTTG 720

GGAAGGGGGG AGCCTGGGGG AAATACTCTA GTGACAACAG TCCTTTCTAA ATCCGGCTGG 780

GGACTGGGTG CAGGTGGGGG TGGGGACGCC CTGCTGCCCC ATATATACAG CCCCTGACCA 840

GGTCAGCTCC ACAGCTCTCT CCCGTGCTCC GTCTTCTTTC CTTGCT 886

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 788 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGCTTGGACG GATAAAGACC TGTCTGGCTC TGGGACTCAC GTGAGTCTCA ACACATCGCC 60

TGCTGATCCA AGGAAATCAG GGCCTGAGCA GAGCGCCCAT GTCTTAGCTC CCTGGTCATC 120

GTCCGCGCCC CTTAGCGAAC AGCTCTCCCT CCAGCTGCCC CCCTCCAGCC CCTGGTTCTG 180

CCCTGCCCTC CCTGGAGCTG AGACTTAGTC CTTTTTCCAT CTCACAGTTG GATGCAGGCT 240

CCAGGCCAGG AAAGCAGGGA AATTTCCCGT TGCAGAAACG GGGCCAGGTC AGCCTTGGAG 300

GCTGGGGGCC TAAGGGGCAG CAGCATGAGG GGCCCACAGT GCCCTGTGGG AGAGCCAGGG 360

CCTGCCTGCC TGAGGTCACA CTGGTGGTCC TGGTCAGTTC CCTCTCCCAC AGGCAGTGGA 420

ATGCGAGGAG ATATTTTTTG CTGCACGTTG AGCCACCCCG CCCCCTGGAA CTCAGACCCT 480

GCACACCCCA TGCCATAACA ATGACGACCA CTTCCAATTG TTTCCTGGCC CGAGGGGGAG 540

GGGAGCTCTC TGGGAGGGGG GGCCCTGGGG GAAATGCTTC CAGTGACAAC AGCCCTTTCT 600

AAATCCGGCT AGGGACTGGG TGCAGGTGGG GGTGGGGGCG CCTGCTGCCC CATATATACA 660

ACCCCTGAGG CCAGGTCTGG CTCTCAGCTC TCTCCTGCTC TCTGTGTCTT TCCTTGATGT 720

TCTCAGGTAG GAGCGGGGAG AAGGGTGGCT CCCAGGTTAG GAAGGGGCTC CCCCAGGAAC 780

AGCAAGCT 788

CLAIMS

1. A recombinant nucleic acid construct comprising:
(i) a mammalian myosin heavy chain promoter;
(ii) nucleic acid encoding a signal sequence; and
(iii) nucleic acid encoding a functional blood clotting protein;
the elements (i)-(iii) being operably linked to provide for expression of the functional protein.

2. A construct according to claim 1 wherein the promoter is a human β-myosin heavy chain promoter.

3. A construct according to claim 1 or 2 wherein the promoter is truncated at between 750 and 850 nucleotides 5' to the start of transcription of the myosin gene from which the promoter is derived.

4. A construct according to any one of the preceding claims which further comprises an enhancer element.

5. A construct according to any one of the preceding claims wherein the signal sequence is the 19 amino acid sequence of Seq. ID. No.2.

6. A construct according to any one of the preceding claims wherein the functional protein is factor VII, VIII, IX or XI.

7. A construct according to claim 6 wherein the factor VIII is a B-domainless factor VIII.

8. A plasmid vector carrying a construct according to any one of claims 1 to 7 together with control sequences for plasmid replication in a host cell.

9. A host cell transformed with a plasmid vector according to claim 8.

10. A pharmaceutical composition comprising a construct according to any one of claims 1 to 7 or a plasmid according to claim 8 in association with a pharmaceutically acceptable carrier or diluent.

11. A construct according to any one of claims 1 to 6 or a vector according to claim 7, or a composition according to claim 10 for use in a method of treatment of the human or animal body.

12. A method of treating a human or animal subject, which subject is suffering from a blood clotting deficiency, which comprises administering to the skeletal muscle of said subject an effective amount of a construct or vector encoding said functional protein, said construct or vector being as defined in any one of claims 1 to 8.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/01114

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/85 C12N15/63 C12N15/90 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 09236 (BAYLOR COLLEGE OF MEDICINE) 13 May 1993 cited in the application See whole document	1,4,8-12
Y	See page 7 to 13 ---	2-7
Y	JOURNAL BIOLOGICAL CHEMISTRY vol. 264, no. 18 , 25 June 1989 , ROCKVILLE PIKE pages 10672 - 10678 L. C. CRIBBS ET AL. 'Muscle-specific regulation of a transfected rabbiit myosin heavy chain beta gene promoter' cited in the application See whole citation --- -/--	2,3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 September 1994	13 -10- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Germinario, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENE & DEVELOPMENT vol. 2 , 1988 pages 1779 - 1790 M. DONOGHUE ET AL. 'A muscle specific enhancer ...' cited in the application See Summary	4
Y	EP,A,0 294 910 (GIST-BROCADES N.V.) 14 December 1988 See Fig 1 and description fig. , page 3	5
A	HUMAN GENE THERAPY vol. 3, no. 1 , February 1992 pages 21 - 33 SHOUSHU JIAO ET AL. 'Direct gene transfer into nonhuman primate myofibers in vivo' See overview summary and page 31, first paragraph lefthand col.	
Y	PROC. NATL. ACAD. SCI. USA vol. 89, no. 8 , 15 April 1992 , WASHINGTON pages 3357 - 3361 SHOU-NAN YAO ET AL. 'Expression of human factor IX in mice after injection in genetically modified myoblasts' See whole citation	6,7

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INTERNATIONAL SEARCH REPORT

...nformation on patent family members

Intn. ...nal Application No
PCT/GB 94/01114

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9309236	13-05-93	US-A-	5298422	29-03-94
		AU-A-	3124693	07-06-93
		CA-A-	2122617	13-05-93
		PT-A-	101042	28-02-94
EP-A-0294910	14-12-88	AU-A-	1809788	04-01-89
		WO-A-	8809813	15-12-88
		US-A-	5171844	15-12-92